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Fluorometric Detection of *B. pertussis* by Polymerase Chain Reaction (PCR) and Molecular Beacon Probe.

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INTRODUCTION

Pertussis is well known as a childhood respiratory disease and is caused by infection with *Bordetella pertussis*. Childhood immunization program for decades has controlled the disease significantly in children. However, studies in civilian and military populations suggest that *B. pertussis* is emerging as a frequently occurring infection among young adults (1-3), due to waning of childhood immunity. Rapid and highly reliable (highly specific and sensitive) diagnosis of the disease is important for providing early appropriate therapeutic intervention, and controlling the disease transmission. Diagnosis by classical laboratory culture method is quite insensitive, depends upon the viability of the bacterial agent, which in turn depends on the specimen collection time during the course of infection, specimen transportation, storage conditions and other unknown factors (4, 5). Diagnosis by other methods such as serology has a higher sensitivity than culture, however serological tests due to necessary time delays are not clinically useful, are not standardized, and not routinely provided by clinical laboratories.

The polymerase chain reaction (PCR) provides an alternative method for rapid detection of a microbial agent in question (6-10). Using the standard PCR, the detection of the amplified DNA fragment with higher specificity and/or sensitivity requires a multistep procedure involving hybridization with a radioactive or non-radioactive probe (9,10). Molecular beacon (11, 12) is a DNA probe system, consisting of a stem-loop structure conjugated with a fluorophore at one end of the stem and a quencher on the other, loop being the probe sequence. The molecular principle of its action is illustrated in Fig. 1.

In brief, the beacon probe changes conformation on homogeneous solution hybridization with its specific target and then emits fluorescence in response to an excitation light. Importantly, the unhybridized fraction of the probe regains its original conformation fast, when the solution containing probe and target DNA is brought from a high hybridization temperature to an appropriate low temperature (lower than the melting temperature of stem structure). The unhybridized probe then does not contribute any fluorescence. The detection of specific PCR product is therefore possible by fluorometry immediately after a target is amplified, in presence of such a probe in the reaction, requiring no additional steps.

The present report is a study on the detection of *B. pertussis* using PCR in presence of a molecular beacon and subsequent fluorescence analysis. The sensitivity of detection by fluorescence analysis was compared with that obtained by agarose gel analysis. This beacon probe based PCR-fluorometric method was also tested in culture positive clinical isolates.

MATERIALS AND METHODS

***B. pertussis* control samples, clinical isolates and nucleic acid (DNA) extraction:** The ATCC

(American Type Culture Collection, Manassas, VA) strain # 9797 of *B. pertussis* was 1:10 serially diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.3) and were used as the control samples. Clinical isolates of *B. pertussis* were provided by the Public Health Laboratory at San Diego, CA. Each isolate was re-grown on Regan-Lowe agar plates. When colonies were visible bacteria from a single colony for each isolate was withdrawn and suspended in 100 μ l of TE buffer. DNA was extracted from each control or clinical sample suspension (100 μ l) using QIamp kit (Quiagen Inc, Chatsworth, CA) following the manufacturer's suggested protocol. The DNA for each sample was eluted in 100 μ l of the manufacturer supplied elution buffer.

Probe design and preparation: The specific molecular beacon probe (5'FAM CCTAGA GATGAACACCCATAAGCATGCCCCG TCTAGG DABCYL 3') was designed with 24 nucleotide long loop sequence (not underlined) complementary to an internal sequence of the DNA fragment to amplify using PCR. The six nucleotide (underlined) sequences formed the stem structure. 5'-6 fluorescein (FAM) and DABCYL were used as the fluorophore and quencher. A commercial company (Synthetic Genetics, San Diego, CA) carried out the synthesis and purification of the beacon probe.

PCR: The sense and antisense primers derived from the tandem repeat sequence region of *B. pertussis* genome were identical to those used in a previously reported study (7). The 100 μ l mixture for PCR in the absence or presence of 0.125 μ M of beacon probe contained, 10 μ l of extracted nucleic acid (DNA), 0.2 mM each of four dNTPs (dATP, dCTP, dGTP, dTTP), 0.3 μ M of each primers, 3.0 mM MgCl₂, and 2.5 U of Taq DNA polymerase in 1X PCR buffer (Perkin Elmer, Inc). The amplification cycle was as follows: 94^o C for 10 sec, 58^o C for 30 sec, and 72^o C for 30 sec. Amplification was performed using 40 cycles. The reaction was subjected to one pre-cycle of 95^o C for 2 min and a post-cycle consisting of 95^o C for 1 min, 70^o C for 2 min, 60^o C for 2 min, 55^o C for 2 min and 50^o C for 2 min and then allowed to cool down to room temperature.

Fluorescence analysis: The fluorescence emission either at fixed wavelength (518 nm, the emission maxima for fluorescein) or spectrum (500-600 nm) was obtained with an excitation at 490 nm using model LS 50B spectrofluorometer (Perkin Elmer). Samples were analyzed either in a micro-cuvette or in multiple well plate readers.

Agarose gel analysis: Five to ten percent of the PCR product were analysed by electrophoresis in TBE buffer (89mM Tris-borate, 2 mM EDTA, pH 8.3) in 2% agarose gel containing 0.5 μ g/ml ethidium bromide. After electrophoresis the DNA bands were visualized through an UV transilluminator.

RESULTS

Molecular Beacon Probe Characterization: The probe solution 50 nM was mixed with 500 nM of complementary oligonucleotide target and incubated at room temperature for 1 hr. The mixed samples were then analyzed for fluorescence emission spectrum. Fig. 2 shows the characteristic emission spectrum of unhybridized (probe alone, curve a) and hybridized samples (curve b). The curve "a" was retraced when the probe was mixed with an oligonucleotide of sequence unrelated to the target. The fluorescence intensity measured at emission maxima at 518 nm, increased about 18-20 fold on hybridization with complementary target. Addition of increased amount of complementary oligonucleotide did not increase the fluorescence further. Also when the probe and

oligonucleotide mixture was heated to 95°C, allowed to cool down to room temperature and then measured the fluorescence, the spectral intensity did not change significantly either (not shown). This verified that all the probes were hybridized with the target, the increase in fluorescence occurred by specific hybridization, and the signal to noise ratio was better than 18:1, for this probe preparation. Also the efficiency of quenching (E_{ff}) of light emitted from fluorophore by the quencher in this probe preparation was found to be > 95% [$E_{ff} = \{1 - (F_{uh} - F_b) / (F_{ch} - F_b)\} \times 100$, where F_{uh} and F_{ch} were the fluorescence intensity of unhybridized and completely hybridized probe respectively. F_b was the background fluorescence of buffer only].

Amplification of control samples of *B. pertussis* and detection: Fig.3 shows the spectrum analysis of *B.pertussis* control samples (10^{-4} to 10^{-9} dilutions) following PCR in presence of the probe. The intensity of fluorescence at 518 nm decreased with increased dilution of sample up to 10^{-8} dilution. The fluorescence intensities of 10^{-8} and 10^{-9} diluted samples were about the same, although there was significant difference in fluorescence from the control negative sample. Therefore maximum sensitivity of detection was assumed to be 10^{-8} dilution. The inset in Fig.3 shows the agarose gel analysis of the amplified DNA fragment for the serial diluted samples. The amplified DNA band intensities decreased gradually from 10^{-4} dilution and the bands were detectable up to 10^{-7} dilution of the sample. The gel electrophoresis data verified that the observed fluorescence of the samples at 518 nm were due the presence of amplified DNA, the amount of which decreased with increased dilution of the sample as expected. Considering the initial titer ($\sim 10^6$ CFU/100 μ l) of the control *B. pertussis* stock, the sensitivity of detection by agarose gel analysis was determined to be < 0.1CFU in the tested volume. The sensitivity of fluorescence analysis was equal or possibly 10 fold better than that obtained by agarose gel analysis.

Amplification of clinical isolates and detection: The PCR amplified product in presence of the beacon probe of fifteen isolates of *B. pertussis* and a control negative sample were analyzed for the fluorescence at 518 nm and the data is provided in Table 1. The fluorescence intensity of all the samples were well above that of the control negative, indicating the presence of amplified *B. pertussis* target gene fragment in all the samples. The specific amplified DNA fragment for each sample was also distinctly detectable by agarose gel electrophoretic analysis (not shown). Thus the result obtained by fluorometric method was in agreement with that of culture and agarose gel analysis.

DISCUSSION AND CONCLUSION

The detection of *B.pertussis* by fluorometry has been demonstrated using PCR and a molecular beacon probe.

The quality and performance efficiency of a molecular beacon probe depends on the signal to noise ratio, and on the efficiency of quenching. The signal to noise ratio (18:1) and the efficiency of quenching (>95%) of the probe used in the present study were comparable to those used in other studies (10-12).

The sensitivity of the fluorometric method was found to be better than 0.1 CFU in the tested volume and was possibly 10 fold higher than that of agarose gel electrophoretic method.

The specificity of the beacon probe and the fluorometric protocol was validated in clinical isolates of *B. pertussis*.

The molecular beacon based method offers a homogeneous solution phase hybridization assay in which amplification and detection of fluorescent signal emitted from the beacon probe hybridized to the amplified DNA can occur in one tube. This makes the assay method easy to perform, and less time consuming by reducing the number of steps compared to other probing methods.

In principle and in practice the risk of carry-over contamination is also considerably minimized by the advantage of performing the entire assay in a hermetically closed tube or micro titer plate.

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Figure Legends:

Fig.1. Schematic diagram of a beacon probe (panel A). S1 and S2 are complementary strands of the stem structure. A fluorophore and a quencher are conjugated to the end of S1 and S2, respectively. The probe sequence is within the loop and is much larger than the stem structure. When such a probe finds its complementary target DNA strand, S1 and S2 are forced apart and the probe-target hybrid is formed. In this conformation of the probe, the quencher (attached to the end of S2) can not adsorb the fluorescence emitted by the fluorophore (attached to the end of S1) and the hybridized probe-target combination will emit fluorescence in response to an excitation light (panel B).

Fig.2. Fluorescence emission spectrum analysis. 50 nM of probe were analyzed for emission spectrum (curve a) before hybridization and (curve b) after hybridization with 500 nM of complementary oligonucleotide, using excitation at 490 nm. The analysis was performed in model LS 50B (Perkin Elmer, Inc).

Fig.3. Fluorescence analysis of serial diluted control samples, following PCR. The dilution of sample was as indicated in each curve. The inset figure shows an agarose gel analysis of the PCR product. Dilution of sample was as shown on top of each lane. Lane M is the 100 bp molecular size standard. Lane N is the control negative PCR.

Table 1. Fluorescence analysis of clinical isolates of *B. pertussis* amplified in presence of the beacon probe.

Sample Number	51	52	53	54	60	63	68	69	71	72	78	81	105	488	9797
Increase in Fluorescence above control negative (ΔF)	37.5	61.4	77.5	87.6	115	74.5	62.4	97.2	85.3	96.2	83.6	89.9	92.0	96.6	99.1

$\Delta F = (F_s - F_n)$, where F_s , and F_n were the fluorescence intensities of sample, and control negative respectively at 518 nm, with excitation at 490 nm. Each F_s value was average of three readings. Average of three F_n values was 481.2. ΔF value of 25 and higher was considered presence of the target and (0 +/- 25) was considered absence of the target in a sample.

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14. ABSTRACT (maximum 200 words)

Bordetella pertussis was detected by spectrofluorometry following PCR incorporating a molecular beacon probe in the reaction. A DNA fragment from the tandem repeat sequence region (IS481) of the genome of *B. pertussis* was amplified in presence of the probe. The probe sequence was complementary to an internal segment of the amplified DNA fragment. Fluorescein (FAM) and DABCYL were used as the fluorophore and quencher in the probe. The probe was characterized for its signal to noise ratio by homogeneous solution hybridization with a complementary oligonucleotide. Measurement of fluorescent signal at the emission maxima of FAM, immediately after a PCR allowed the detection of a *B. pertussis* target, requiring no additional steps. Presence of *B. pertussis* in a sample was also examined by agarose gel electrophoresis following PCR. A serial diluted stock of ATCC strain #9797 of *B. pertussis* and fourteen clinical isolates of *B. pertussis* were examined. The sensitivity of detection by fluorescent measurement was found to be at least in the range of 0.01- 0.1 CFU in the tested volume of the sample and it was equal to or better than that detected by agarose gel analysis.

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